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A Role for Cyclin E/Cdk2 in the Timing of the Midblastula Transition in *Xenopus* Embryos

Rebecca S. Hartley, Jill C. Sible, Andrea L. Lewellyn, and James L. Maller[1]

Howard Hughes Medical Institute and Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262

During *Xenopus* development, the early cell cycles consist of rapid oscillations between DNA synthesis and mitosis until completion of the 12th mitotic division. Then the cycle lengthens and becomes asynchronous, zygotic transcription begins, and G phases are established, a period known as the midblastula transition (MBT). Some aspects of the MBT, such as zygotic transcription, depend on acquisition of a threshold nuclear to cytoplasmic (N/C) ratio, whereas others, such as maternal cyclin E degradation, are independent of nuclear events and appear to be controlled by an autonomous maternal timer. To investigate the function of cyclin E during the early cycles, cyclin E/Cdk2 kinase activity was specifically inhibited in fertilized eggs by a truncated form of the *Xenopus* Cdk inhibitor, Xic1 (Δ34Xic1). Δ34Xic1 caused lengthening of the embryonic cell cycles that correlated with increased levels of mitotic cyclins. However, DNA synthesis was not inhibited. Several hallmarks of the MBT were delayed for several hours in Δ34Xic1-injected embryos, including the disappearance of cyclins E and A, the initiation of zygotic transcription, and the reappearance of phosphotyrosine on Cdc2. In both control and Δ34Xic1-injected embryos, cyclin E was degraded after the 12th mitotic division as zygotic transcription began, but experiments with α-amanitin show that cyclin E degradation is not dependent on zygotic transcription. Thus, the length of the early cycles and the timing of maternal cyclin degradation depend upon cyclin E/Cdk2 activity. Neither oscillations in cyclin E/Cdk2 activity during the early cycles nor the disappearance of cyclin E at the MBT were dependent on protein synthesis. These data suggest that cyclin E/Cdk2 is directly linked to an autonomous maternal timer that drives the early embryonic cell cycles until the MBT. q **1997 Academic Press**

INTRODUCTION

Changes in the cell cycle are key features of important developmental transitions during determination and differentiation. We have been studying developmental regulation of the cell cycle in *Xenopus* embryos both during the rapid, synchronous cleavage divisions and in the period of cell cycle remodeling that occurs at the midblastula transition (MBT) (Rempel *et al.,* 1995; Hartley *et al.,* 1996). During cleavage divisions, which consist solely of mitosis and S-phase, synthesis and accumulation of B cyclins drive formation of Cdc2 complexes and entry into mitosis, and cyclin B degradation results in exit from mitosis (Murray and Kirschner, 1989; Hartley *et al.,* 1996). In contrast, the timing of cyclin E accumulation and its associated Cdk2 activity differ from those of the B-type cyclins. In adult somatic cells, cyclin E is periodically expressed and maximally activates Cdk2 at the G_1/S transition (Dulic *et al.,* 1992; Koff *et al.,* 1992), implying a function in S-phase. In *Xenopus* embryos, cyclin E protein accumulates during meiosis II and the first mitotic cycle, after which its level remains constant until the MBT (Rempel *et al.,* 1995; Hartley *et al.,* 1996). Although cyclin E protein level does not vary, cyclin E/Cdk2 activity does vary, with peaks at both mitosis and S-phase in each cycle. The S-phase peak may indicate a role for cyclin E/Cdk2 in embryo DNA synthesis, but studies in cell-free extracts show that cyclin A/Cdc2, which is also present in the embryo, is a more potent mediator of DNA synthesis (Strausfeld *et al.,* 1994, 1996). The M-phase peak in embryos suggests a role for cyclin E/Cdk2 in initiation of mitosis, and recent studies on mitotic initiation in egg extracts lacking DNA confirm this idea (Guadagno and Newport, 1996). Upon completion of the 12th mitotic division, cyclin E1 protein is degraded (Rempel *et al.,* 1995; Hartley *et al.,* 1996). This degradation of cyclin E1 at the MBT occurs during the transition from maternal to zygotic control, when S-phase lengthens and G phases are added to

[1] To whom correspondence should be addressed.

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the simple biphasic cycle. Some events of the MBT, such as zygotic transcription, appear to depend on acquisition of a threshold nuclear to cytoplasmic (N/C) ratio, whereas others appear to be controlled by a timing mechanism activated at fertilization. Howe and Newport (1996) have proposed that this developmental timing mechanism controls the degradation of cyclin E because this event is independent of cell cycle progression, new protein synthesis, or the N/C ratio, features reminiscent of the cytoplasmic oscillator first proposed by Hara and colleagues (1980). The work in this paper was designed to evaluate the role of cyclin E1/Cdk2 in the early divisions and at the MBT. These studies have taken advantage of the *Xenopus* cdk inhibitor, Xic1 (Su *et al.*, 1995). Full-length Xic1 inhibits cyclin E/Cdk2 most efficiently, but it also inhibits cyclin A/ and B/cdk complexes at higher IC_{50} values. However, a truncated form of Xic1, in which the first 34 amino acids have been deleted (Δ34Xic1), is highly specific for cyclin E/Cdk2 (Su *et al.*, 1995). Specific inhibition of cyclin E/Cdk2 complexes in newly fertilized eggs has allowed selective dissection of cyclin E/Cdk2 function in early embryonic cell cycles and provides evidence that this Cdk complex is involved in controlling both the length of the early cycles and the timing of the MBT.

MATERIALS AND METHODS

Embryos and Microinjections

Embryos were obtained by *in vitro* fertilization, dejellied in 2% cysteine (pH 7.8), cultured in 0.1× MMR, and staged according to Nieuwkoop and Faber (1975). When indicated, freshly prepared cycloheximide (CHX) or aphidicolin was added to the culture medium at a final concentration of 100 μg/ml. Enhanced uptake of these compounds was obtained by incubating embryos in Danilchik's solution (Peng, 1991) containing 5% Ficoll. For time-course studies, embryos were collected at the indicated times, frozen on dry ice, and stored at −807C. Embryos were homogenized in 10 vol of extraction buffer (EB) and microcentrifuged for 5 min to remove insoluble and yolk proteins, and the supernatants used for immunoprecipitation, *in vitro* kinase assays, and immunoblots. EB is composed of 20 m*M* Hepes (pH 7.5), 80 m*M* β-glycerophosphate, 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 50 m*M* NaF, 1 m*M* sodium vanadate, 3 μg/ml leupeptin, 0.5 m*M* phenylmethylsulfonyl fluoride, 0.2 m*M* ammonium molybdate, 30 m*M* *p*-nitrophenyl phosphate, and 1 μ*M* microcystin.

Glutathione *S*-transferase (GST)-tagged fusion proteins of full-length Xic1, Δ34Xic1 (a mutant lacking the first 34 amino acids in the highly conserved cdk-binding domain), and C-Xic1 (an N-terminal truncation containing only C-terminal residues 97–210) were prepared as described previously (Su *et al.*, 1995). Unless otherwise indicated dejellied embryos were manually injected with 5 ng of GST–Δ34Xic1 or with buffer (20 m*M* Hepes, 88 m*M* NaCl, 7.5 m*M* $MgCl_2$, 20 m*M* β-mercaptoethanol, 5% glycerol). This results in a final intracellular concentration of 185 n*M* Δ34Xic1, a level that maximally inhibits cyclin E/Cdk2 complexes without affecting other Cdks (Su *et al.*, 1995 and Fig. 1). Full-length Xic1 at 5 ng inhibited not only cyclin E/Cdk2 but also cyclin A/Cdc2 and cyclin B/Cdc2, whereas Δ34Xic1 began to inhibit Cdc2 complexes only at levels of 10 ng or more, and C-Xic1 did not cause any inhibition of cyclin/cdk complexes (data not shown). In some experiments, embryos were injected with α-amanitin to a final concentration of 50 μg/ml in the embryo (50 nl of 1 mg/ml). Microinjections were performed at the one-cell stage between 40 and 70 min postfertilization, and embryos were maintained in a solution of 0.1× MMR containing 5% Ficoll.

Antibodies and Immunoblot Analysis

Antisera were raised in sheep against full-length *Xenopus* cyclins A1, B1, and B2, as described by Gautier *et al.* (1990). Cyclin E1 antiserum was raised in a goat against full-length recombinant *Xenopus* cyclin E1 (Rempel *et al.*, 1995). Antiphosphotyrosine antibodies were the kind gift of Dr. Wilfried Merlevede (Katholieke Universiteit te Leuven, Belgium). For immunoblots, antisera to cyclins E1, B1, and B2 were blot-purified, and cyclin A1 antiserum was affinity-purified, as described previously (Gautier *et al.*, 1990; Rempel *et al.*, 1995). For immunoblot analysis, extracts were subjected to electrophoresis on SDS–polyacrylamide gels and transferred to supported nitrocellulose membranes using a semidry blotting apparatus (LKB). Membranes were blocked with 10% nonfat dry milk in PBS and incubated for 1 hr at room temperature with primary antibody diluted in 10% nonfat dry milk in PBS. Membranes were then washed with PBS 0.5% Tween 20, incubated in the appropriate secondary antibody conjugated to peroxidase (Jackson ImmunoResearch) diluted 1:5000 in 10% nonfat dry milk in PBS, washed as above, and developed by enhanced chemiluminescence (Amersham).

Immunoprecipitation

Immunoprecipitation of cyclin-associated kinase activity was performed essentially as described previously (Gabrielli *et al.*, 1992). Briefly, egg or embryo extracts were diluted in EB to a final volume of 100 μl and precleared with protein G–agarose in EB. Precleared samples were separated into three equal aliquots and incubated with cyclin E1 antiserum, affinity-purified cyclin A1 antiserum, or a mixture of cyclin B1 and cyclin B2 antisera for 1 hr on ice. Twenty-five microliters of 50% protein G–agarose in EB was then added and samples were mixed for 1 hr at 47C on a rotator, washed 2× with low- and high-salt buffers (20 m*M* Tris-HCl, pH 7.4, 5 m*M* EDTA, 0.1% Triton X-100, containing either 100 m*M* or 1 *M* NaCl, respectively), and finally washed with kinase assay buffer (20 m*M* Hepes, pH 7.5, 15 m*M* $MgCl_2$, 5 m*M* EGTA, 1 m*M* DTT). Immunoprecipitates were incubated in 25 μl of kinase assay buffer containing 0.2 mg/ml BSA, 1.0 mg/ml histone H1, and 200 μ*M* [γ-^{32}P]ATP (2 cpm/fmole). Samples were incubated at 257C for 20 min, and the reactions were stopped by the addition of 10 μl of 5× sample buffer. Samples were heated at 957C for 2 min, electrophoresed on 12.5% Laemmli polyacrylamide gels, and phosphorylation of histone H1 was quantified by scintillation counting of the excised H1 band.

Northern Analysis and DNA Synthesis Assay

Embryos were collected for Northern analysis of GS17 expression and for immunoblot analysis when buffer-injected embryos reached Stages 8, 9, 10, and 10.5. Total RNA was extracted from 20 embryos per stage using the TRIreagent protocol (Molecular Resource Center, Inc.). Briefly, embryos were homogenized in TRI-

reagent and extracted with chloroform, and the RNA was precipitated with isopropanol. Ten micrograms of RNA per lane was electrophoresed on a 1.2% denaturing formaldehyde gel and transferred to a nylon membrane by capillary action. Membranes were hybridized with Quikhyb according to the manufacturer's protocols (Stratagene) and probed with a GS17 cDNA probe (Krieg and Melton, 1985).

For analysis of DNA synthesis, injected embryos were incubated until the 32- to 64-cell stage (about 1.5 hr) in 800 μCi/ml [^{3}H]-thymidine (sp act 64 Ci/mmole) diluted in Danilchik's medium containing 5% Ficoll. Embryos were then transferred to 0.1× MMR with 5% Ficoll and 10 embryos were collected every 30 min until 5 hr postfertilization. DNA was extracted and incorporation of [^{3}H]-thymidine was measured by TCA precipitation and scintillation counting (Newport and Kirschner, 1982).

RESULTS

Δ34Xic1 Inhibits Cyclin E/Cdk2 and Slows the Cell Cycle

As described in the Introduction, the autonomy of cyclin E degradation suggests control of this event by the proposed developmental timer that regulates the oscillatory pre-MBT cell cycle (Howe and Newport, 1996). If cyclin E/Cdk2 is indeed a component of the developmental timer, then perturbation of cyclin E activity should alter the mitotic cell cycle and/or the timing of the MBT. To study cyclin E/Cdk2 function, embryos were injected with 5 ng of a truncated form of GST-tagged Xic1 (Δ34Xic1), a *Xenopus* cdk inhibitor that specifically inhibits the activity of *Xenopus* cyclin E1/Cdk2 complexes at a low concentration that does not affect other cyclin/cdk complexes (Su *et al.*, 1995). Embryos were injected at the 1-cell stage with buffer, Δ34Xic1, or a mutant containing only the noninhibitory C-terminal half of Xic1 (C-Xic1) and then incubated until buffer-injected (control) embryos reached Stage 8. Figure 1A shows that 6 hr after fertilization, control embryos had reached Stage 8 (approximately 4000 cells), whereas Δ34Xic1-injected embryos had fewer and larger cells, resembling early Stage 7 embryos (Fig. 1A). Embryos injected with the noninhibitory C-Xic1 or with an equimolar mixture of Xic1 and cyclin E/Cdk2 resembled control embryos (data not shown). Dose response curves indicated that injection of at least 2 ng Xic1 was necessary to inhibit cyclin E/Cdk2 by >80% and observe slowing of the cell cycle (data not shown). Thus, embryos in which cyclin E/Cdk-2 is inhibited undergo fewer cell divisions than control embryos in the same time period.

To measure directly the inhibition of cdks after Δ34Xic1 injection, cyclins were also immunoprecipitated from injected embryos during the second and third cycles to assay their associated kinase activities. Figure 1B shows that cyclin E/Cdk2 activity was inhibited by 80–90% throughout early cycles in Δ34Xic1-injected embryos. Although the magnitude of mitotic cyclin A1/Cdc2 kinase activity was not inhibited, there was a delay in the timing of each cycle of kinase activity (Fig. 1C). A similar delay was evident in the timing of each cycle of cyclin B/Cdc2 activity (data not shown). Cytokinesis, as indicated by the arrows in Figs. 1B and 1C, was also delayed in Δ34Xic1-injected embryos. In any particular cycle, the cleavage delay was between 0 and 10 min, but on average, the length of each cell cycle was increased approximately 20% (5 min) in Δ34Xic1-injected embryos. This is consistent with the delay seen in mitotic peaks of Cdc2 activity (Fig. 1C) and results in fewer cell divisions in a given period of time (Fig. 1A).

Degradation of Cyclins E1 and A1 Is Delayed in Δ34Xic1-Injected Embryos

Until the MBT, which begins when the embryo has undergone 12 divisions, the steady-state level of cyclin E1 is constant. Upon completion of the 12th mitotic division, cyclin E1 protein is abruptly degraded and Cdc2 becomes phosphorylated on tyrosine residues (Rempel *et al.*, 1995; Hartley *et al.*, 1996). Cyclin A1 is also degraded, but not until gastrulation (Howe *et al.*, 1995; Rempel *et al.*, 1995; Hartley *et al.*, 1996). It has been reported that cyclin E degradation occurs even when the mitotic cell cycle is absent due to inhibition of cyclin B synthesis by CHX (Howe and Newport, 1996). Although this suggests that the timing of degradation does not require a mitotic cell cycle, it does not rule out an effect of an active mitotic cycle on the timing of cyclin E degradation or the necessity of cyclin E/Cdk2 activity for cyclin E degradation. To determine whether cyclin E degradation is dependent on the activity of cyclin E1/Cdk2, on the number of cell divisions embryos have undergone, or on the amount of time elapsed since fertilization, embryos were injected with either Δ34Xic1 or buffer, and development was monitored. Embryos were also collected every 15–30 min and immunoblotted with antisera specific for cyclin E, cyclin A1, or phosphotyrosine (Fig. 2). Cyclin E was degraded at 7 hr postfertilization in buffer-injected embryos, whereas in Δ34Xic1-injected embryos, cyclin E was present until 9 hr postfertilization (Fig. 2), despite the fact that cyclin E/Cdk2 kinase activity was low. In addition, a faster migrating, hypophosphorylated form of cyclin E predominated in Δ34Xic1-injected embryos, providing further evidence that Δ34Xic1 inhibited the activity of cyclin E/Cdk2 complexes even 8 hr after injection, since complexes with the hypophosphorylated form of cyclin E are kinase inactive (Rempel *et al.*, 1995). The delayed degradation of cyclin E at 9 hr in the Δ34Xic1-injected embryos corresponded to the time of the 12th mitotic division and apparently did not require cyclin E/Cdk2 activity.

Similar to the results with cyclin E, the time at which cyclin A1 disappeared in Δ34Xic1-injected embryos was also delayed (Fig. 2, cyclin A1). In control embryos, the level of cyclin A1 declined in the early gastrula, beginning around 8 hr 30 min postfertilization. In Δ34Xic1-injected embryos, cyclin A1 was degraded at 9 hr simultaneously with cyclin E1. There was also a lag in the reappearance of phosphotyrosine on Cdc2 in Δ34Xic1-injected embryos compared to buffer-injected embryos (Fig. 2B, phosphotyrosine). A strong

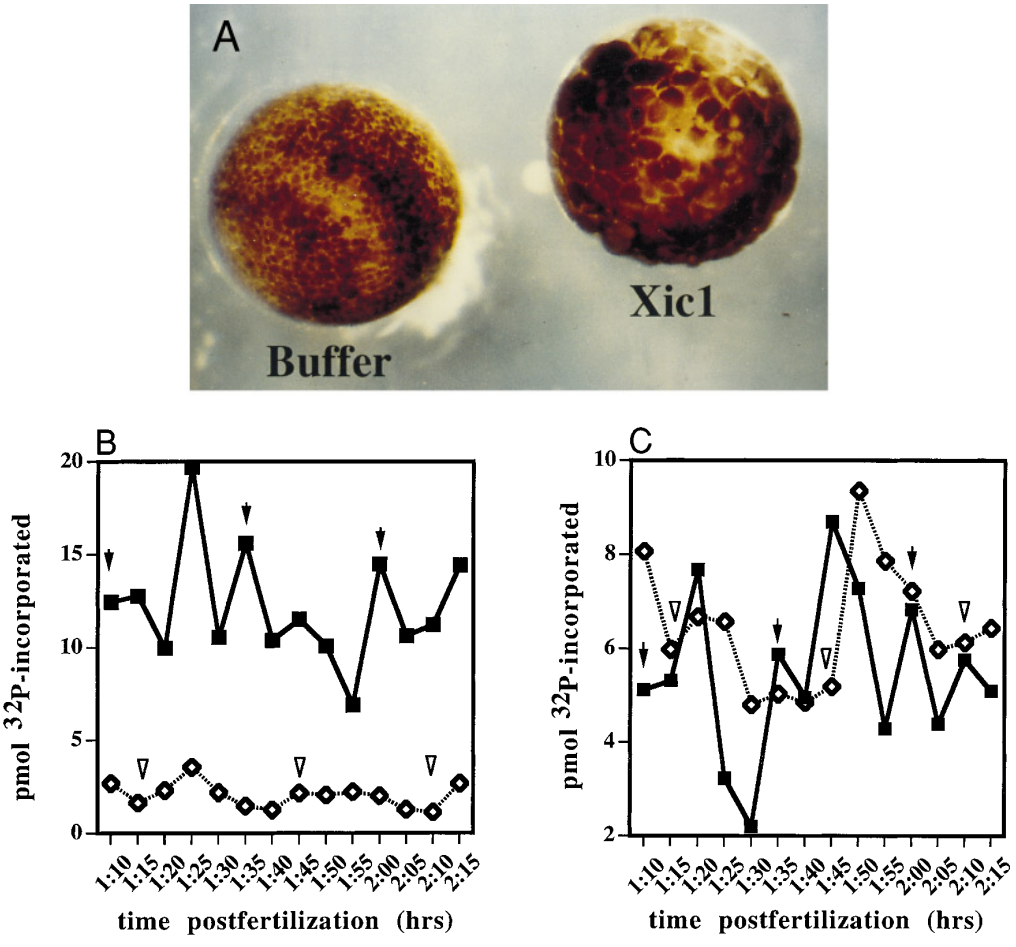

FIG. 1. Δ34Xic1 slows cleavage divisions and specifically inhibits cyclin E/Cdk2 complexes in the early *Xenopus* embryo. (A) Embryos were injected at the 1-cell stage with buffer or Δ34Xic1 and allowed to develop until buffer-injected embryos reached Stage 8 (6 hr postfertilization). (B) At the indicated times, histone H1 kinase activity was measured in immunocomplexes with antibodies against cyclin E1. (C) In the same experiment as that in B, cyclin A/Cdc2 complexes were assayed for H1 kinase activity in immunocomplexes. Previous studies show that cyclin E is bound only to Cdk2 and cyclin A only to Cdc2 (Rempel *et al.*, 1995). The equivalent of two embryos was immunoprecipitated with each antiserum. The closed arrows represent cytokinesis in buffer-injected embryos; the open arrowheads, cytokinesis in Δ34Xic1-injected embryos. (■) buffer-injected; (└) Δ34Xic1-injected.

phosphotyrosine signal appeared on Cdc2 at about 7 hr postfertilization in buffer-injected embryos, but not until 8 hr 15 min in Δ34Xic1-injected embryos. These data indicate that the developmental timer (Howe and Newport, 1996) does not merely measure time elapsed after fertilization before initiation of the MBT and degradation of maternal cyclins. In fact, the data suggest an integral part of this timer may monitor cyclin E/Cdk2 activity.

The Timing of Zygotic Transcription Depends upon Cyclin E/Cdk2 Activity

The timing of one hallmark of the MBT, the initiation of zygotic transcription, may depend on either a developmental timer that monitors time elapsed post-fertilization or the nuclear to cytoplasmic ratio (Newport and Dasso, 1989). Because maternal cyclin degradation and zygotic transcription occur at the same time, it is not known if initiation of zygotic transcription requires cyclin E degradation and disengagement from the maternal cell cycle; therefore, we asked whether the delayed degradation of cyclin E protein following injection of Δ34Xic1 would alter the timing of zygotic transcriptional initiation. Transcription was monitored by the appearance of GS17, a gastrula-specific transcript expressed only after the MBT (Krieg and Melton, 1985. Northern analysis of control embryos showed that GS17 was expressed at Stage 9, with increased expression by Stage 10 (Fig. 3A, buffer). Significantly, GS17 expression was delayed in Δ34Xic1-injected embryos until control embryos reached Stage 10 (Fig. 3A, Δ34Xic1). These results

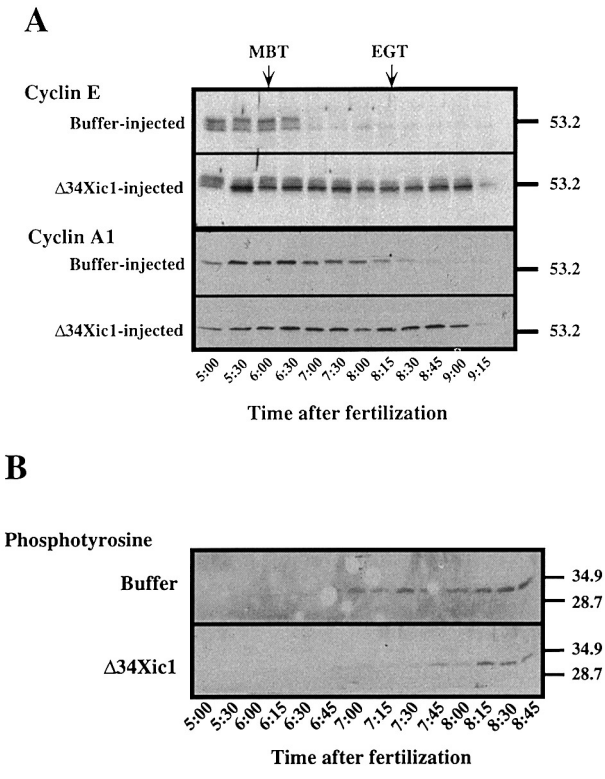

FIG. 2. Effect of Δ34Xic1 on cyclin degradation and the reappearance of phosphotyrosine on Cdc2 at the MBT. (A) Embryos were injected at the 1-cell stage with buffer or Δ34Xic1 and embryos collected every 15–30 min between 5 and 9 hr postfertilization. Immunoblots were performed using specific antibodies to cyclin E, cyclin A1, and phosphotyrosine, as indicated. The blot was probed for cyclin E, stripped and reprobed for cyclin A1. The equivalent of one embryo was loaded per lane. MBT and EGT are the midblastula and early gastrula transitions, respectively. (B) The phosphotyrosine content of Cdc2 was monitored by immunoblotting extracts from control (buffer-injected) and Δ34Xic1-injected oocytes. Previous data demonstrate the phosphotyrosine band shown is Cdc2 (Hartley *et al.*, 1996). Molecular weight markers in kDa are indicated on the right of each panel.

indicate that the initiation of zygotic transcription does not involve a mechanism that measures elapsed time postfertilization but rather coincides with and may depend upon degradation of maternal cyclins.

In both buffer- and Δ34Xic1-injected embryos, cyclin E protein disappeared coincident with expression of GS17 (Fig. 3B). In addition, there was an increase in cyclin B1 and cyclin B2 upon initiation of zygotic transcription that correlated with the abrupt decrease in cyclin E. The increase in cyclin B was delayed in Δ34Xic1-injected embryos, an increase which could be due to either increased synthesis of the maternal protein or expression of zygotic forms which cross-react with the antibodies used. Accumulation of the B cyclins could also be a consequence of cell cycle remodeling at the MBT, which initially extends the length of S-phase of the cycle, when cyclin B is synthesized (Frederick and Andrews, 1994).

Since the decrease in cyclin E corresponded closely with upregulation of GS17, we asked whether the degradation of cyclin E is dependent on zygotic transcription. Embryos were injected at the 1-cell stage with α-amanitin, an inhibitor of RNA polymerase, which completely inhibited expression of GS17 (not shown). Embryos were collected every 30 min from 5 to 11 hr postfertilization and blotted for cyclins E and B. Cyclin E was degraded with the same kinetics in α-amanitin- and buffer-injected embryos, and the initial increase in cyclin B2 at Stage 9 was also unaffected by α-amanitin (Fig. 4). Other experiments indicate the continued increase in cyclin B2 expression after 10 hr in α-amanitin-injected embryos coincides with induction of apoptosis (unpublished data). Therefore, it appears that changes in both cyclins E and B at the MBT are controlled by a distinct maternal program that requires no zygotic input, but the initiation of zygotic transcription may be regulated by this maternal program.

Δ34Xic1 Does Not Inhibit DNA Synthesis in Vivo but Does Affect the Timing of Mitosis

We previously demonstrated that in the early cell cycles there are two peaks of cyclin E/Cdk2 activity, one in S-phase and one in M-phase (Hartley *et al.*, 1996). Therefore, lengthening of the cell cycle by inhibition of cyclin E/Cdk2

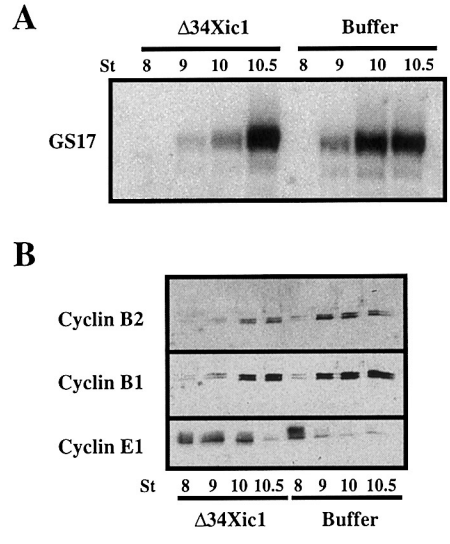

FIG. 3. Zygotic transcription correlates temporally with degradation of cyclin E1. (A) RNA was isolated from buffer- or Δ34Xic1-injected oocytes at the indicated stages and Northern analysis performed with a probe specific for GS17, a gastrula-specific transcript expressed only after the MBT. (B) Western analysis on embryos from the same experiment as (A) showing that the expression of GS17 correlates temporally with the decline in cyclin E1 and an increase in cyclin B1 and cyclin B2.

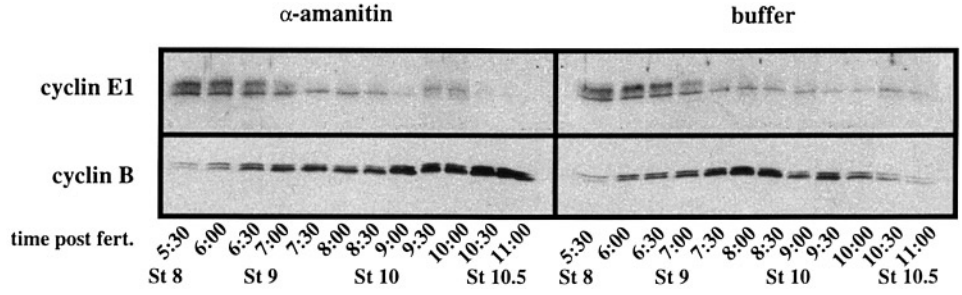

FIG. 4. Degradation of cyclin E1 at the MBT is independent of zygotic transcription. Embryos were injected with the transcriptional inhibitor α-amanitin at the 1-cell stage, collected at the indicated times, and blotted for cyclin E. The same blot was stripped and reprobed for cyclin B2.

could be due to lengthening of DNA synthesis or mitosis or to introduction of a G-phase. In *Xenopus* egg extracts, depletion of Cdk2 blocks DNA synthesis, suggesting that Cdk2 has a role in regulating this process (Blow and Nurse, 1990; Fang and Newport, 1991; Strausfeld *et al.*, 1996). Moreover, both cyclin A/Cdc2 and *Xenopus* cyclin E/Cdk2 can rescue inhibition of DNA synthesis resulting from Cdk2 depletion or from addition of the human cdk inhibitor, p21Cip1, to *Xenopus* egg extracts, with cyclin A/Cdc2 being more efficient in this process (Strausfeld *et al.*, 1994, 1996; Su *et al.*, 1995). We examined DNA synthesis in Δ34Xic1-injected embryos by measuring incorporation of [^{3}H]thymidine into DNA. Immediately after injection, embryos were incubated in [^{3}H]thymidine for 1.5 hr (approximately the 2- to 32-cell period), then removed from label, and collected every 30 min from the 32-cell stage through Stage 8. There was no significant inhibition of DNA synthesis by Δ34Xic1 relative to controls (Fig. 5A). DAPI staining of nuclei showed the expected number and size of nuclei in Δ34Xic1-injected embryos as well as normal chromosome morphology (data not shown). These results suggest that the cell cycle lengthening observed in Δ34Xic1-injected embryos is not due to inhibition of S-phase or failure to limit DNA replication to one round per cell cycle.

Because the cell cycle is lengthened only 20% by inhibition of cyclin E/Cdk2, it is not certain thymidine incorporation data would reveal an increase in the length of S-phase. Therefore, the effect of Δ34Xic1 was monitored in embryos incubated in the presence of aphidicolin to inhibit DNA synthesis. The concentration of aphidicolin needed to completely inhibit DNA synthesis was higher than previously reported (Newport and Dasso, 1989). With complete inhibition of DNA synthesis, the cell cycle, as monitored by cytokinesis, was somewhat delayed by aphidicolin, but cytokinesis occurred normally through the MBT (Fig. 5B). The delay in cytokinesis seen with aphidicolin could represent partial activation of a DNA replication checkpoint in the cell cycle. Importantly, however, even in the presence of aphidicolin, the cell cycle was further lengthened in embryos injected with Δ34Xic1 (Fig. 5B). The additive effects of aphidicolin and Δ34Xic1 suggest that the lengthening of the cell cycle by inhibition of cyclin E/Cdk2 occurs by a mechanism independent of inhibition of DNA synthesis.

In part because cyclin E/Cdk2 activity also peaks near M-phase (Rempel *et al.*, 1995; Hartley *et al.*, 1996), we examined levels of mitotic cyclins to determine whether mitosis was affected by inhibition of cyclin E/Cdk2. Embryos injected with Δ34Xic1 at the 1-cell stage were collected during the first two cycles after injection (2- to 8-cell, Fig. 6A), and immunoblotted. Increased levels of mitotic cyclins were observed in Δ34Xic1-injected embryos compared to controls prior to and at the peak of M-phase H1 kinase activity (cf. Fig. 1). The higher levels of mitotic cyclins did not result from decreased cyclin turnover, since the degradation of mitotic cyclins was not delayed in Δ34Xic1-injected embryos incubated in CHX to inhibit resynthesis of cyclins (Fig. 6B). Therefore, these results suggest that inhibition of cyclin E/Cdk2 by Xic1 results in an extended S to M transition, presumably by affecting an essential step required for entry into M-phase (Guadagno and Newport, 1996).

Oscillations in Cyclin E/Cdk2 Activity Are Independent of Protein Synthesis

Protein synthesis is required for normal progression through the cell cycle. During the cleavage divisions the only essential newly synthesized protein is cyclin B (Murray and Kirschner, 1989; Hartley *et al.*, 1996). Incubation of embryos in CHX (100 μg/ml) inhibits protein synthesis, thus halting mitotic cell cycle progression (Minshull *et al.*, 1989; Murray and Kirschner, 1989). The timing of degradation of cyclin E is not affected by CHX, suggesting that this proteolytic program is independent of protein synthesis (Howe and Newport, 1996). Since the level of cyclin E does not oscillate during the cell cycle (Rempel *et al.*, 1995; Hartley *et al.*, 1996) and inhibition of cyclin E/Cdk2 activity changes the timing of maternal cyclin degradation and the initiation of zygotic transcription, we examined the effect of inhibition of protein synthesis on the activity of cyclin E/Cdk2 complexes. In the presence of CHX, mitotic cyclins were degraded and not resynthesized (data not shown) and associated Cdc2 activity was inhibited (Fig. 7A). In contrast,

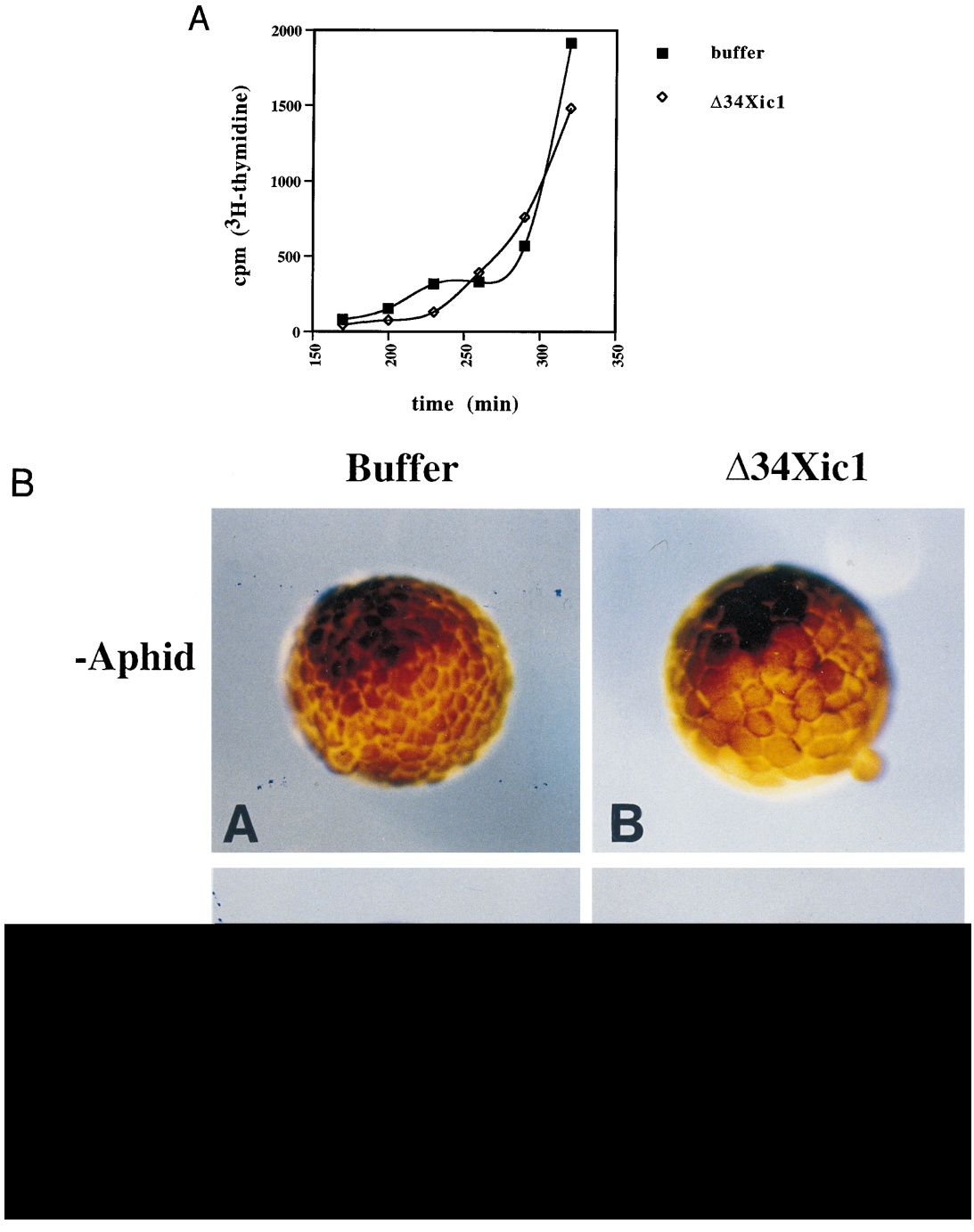

FIG. 5. Inhibition of cyclin E1/Cdk2 does not inhibit embryonic DNA synthesis. (A) Embryos injected at the 1-cell stage with either buffer or Δ34Xic1 were incubated in [^{3}H]thymidine until the 32- to 64-cell stage. Ten embryos were then collected every 30 min and subsequently assayed for incorporation of radiolabel into DNA by TCA precipitation. (B) Embryos injected with either buffer or Δ34Xic1 were incubated with 100 μg/ml aphidicolin (+Aphid) or not (−Aphid) and allowed to develop until control (−Aphid) embryos reached early Stage 8.

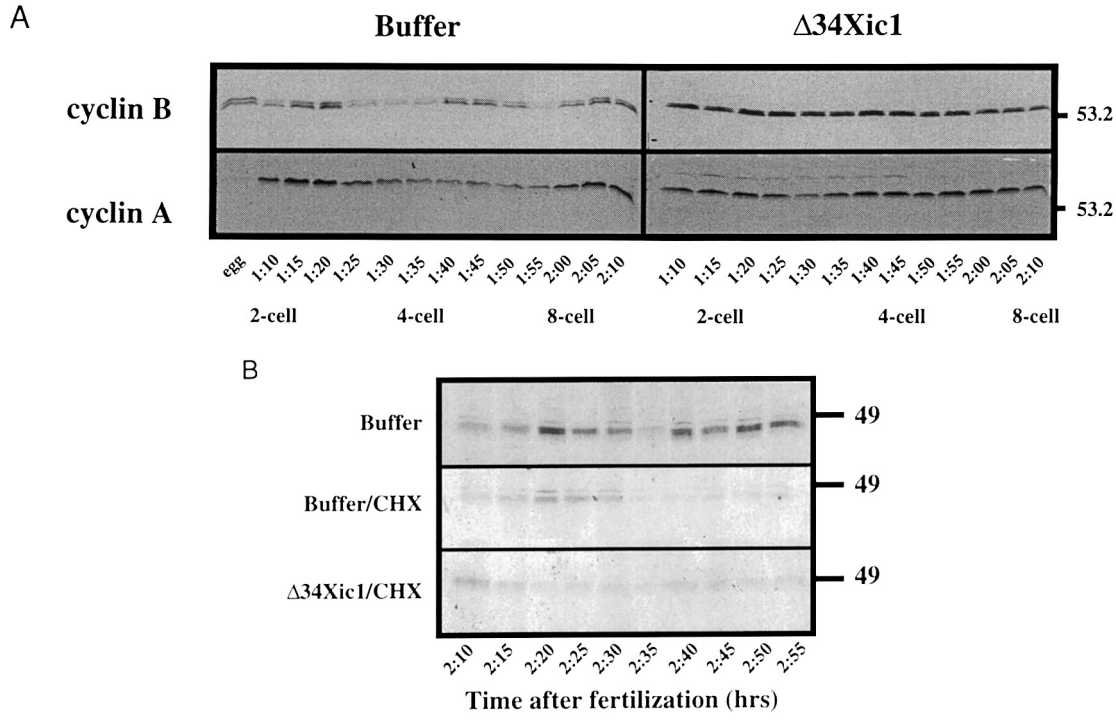

FIG. 6. Levels of mitotic cyclins are increased in Δ34Xic1-injected embryos. (A) Western blot showing cyclin levels during the first two cell cycles following Δ34Xic1 injection (2- to 8-cell). One embryo equivalent was loaded per lane, and the same blot was probed successively for cyclins B1 and A1. (B) Cyclin B2 blot of embryos injected with buffer or Δ34Xic1 and incubated in CHX (100 μg/ml) immediately after injection. Molecular weight markers in kDa are indicated on the right of each panel.

the level of cyclin E remained constant (data not shown), and Cdk2 activity still oscillated (Fig. 7B). Thus, cyclin E/Cdk2 oscillations are independent of protein synthesis and proceed even in the absence of cell division, which suggests a role for cyclin E/Cdk2 in a developmental timer that is independent of mitotic cell cycle progression.

DISCUSSION

Cyclin E/Cdk2 has an established role in higher eukaryotes in regulating the G_1/S transition. The results in this paper demonstrate that cyclin E/Cdk2 is also required for timing the early embryonic cell cycles *in vivo*. In support of this, we have shown that specific inhibition of cyclin E/Cdk2 activity results in lengthening of the early embryonic cell cycle. Associated with these extended cell cycles is higher accumulation of mitotic cyclins, although Cdc2 activity at metaphase is of normal magnitude despite higher mitotic cyclin levels. The delay in entry into mitosis as judged by Cdc2 H1 kinase activity in Δ34Xic1-injected embryos is consistent with delayed cytokinesis and the observed slower cell cycle. A role for cyclin E/Cdk2 in mitosis was suggested by earlier studies that showed high levels of cyclin E/Cdk2 activity during M-phase in embryos and in egg extracts (Rempel *et al.*, 1995; Hartley *et al.*, 1996). More recently, *in vitro* studies using cdk inhibitors in egg extracts lacking DNA (Guadagno and Newport, 1996) have shown a requirement for cyclin E/Cdk2 for correct timing of entry into mitosis. This suggests that the cell cycle delay seen when cyclin E/Cdk2 is inhibited is due to interference with initiation of M-phase, resulting in continued mitotic cyclin accumulation.

Inhibition of cyclin E/Cdk2 activity delayed the degradation of cyclin E and cyclin A1, the reappearance of phosphotyrosine on Cdc2, and the initiation of zygotic transcription. These results are consistent with the embryo having completed fewer cell cycles compared to control embryos in the time elapsed postfertilization and suggest that cyclin E/Cdk2 activity is necessary for correct timing of maternal cyclin degradation and transcriptional initiation. This timing mechanism is clearly not simply a measure of N/C ratio, since neither inhibition of DNA synthesis by aphidicolin nor inhibition of the cell cycle by CHX affects the timing of degradation of cyclin E (Howe and Newport, 1996, and our unpublished data). In view of the fact that both peaks of cyclin E/Cdk2 activity are independent of new protein synthesis, DNA synthesis, the N/C ratio, cell cycle progression, and new transcription, all proposed attributes of the developmental timer which controls the MBT, we suggest

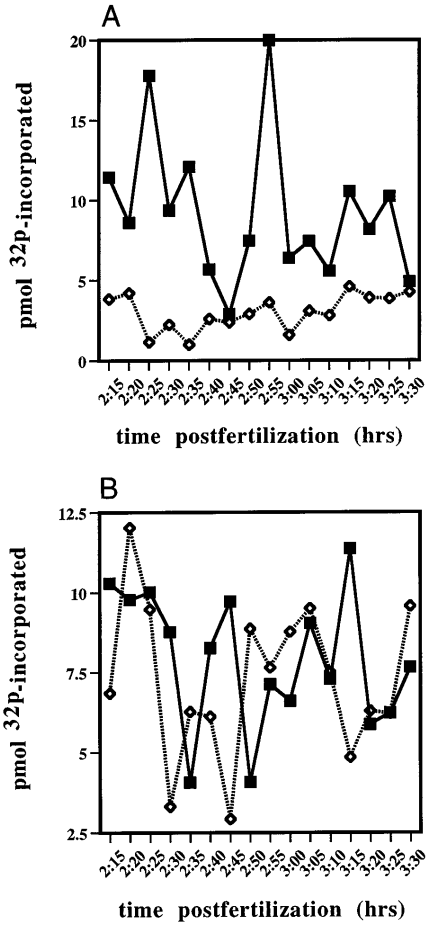

FIG. 7. Oscillations in cyclin E/Cdk2 activity are not dependent on protein synthesis. Histone H1 kinase activity was immunoprecipitated from embryos incubated in the presence (L) or the absence (■) of CHX. Embryos were immunoprecipitated with cyclin B antibodies (A) or with cyclin E antibodies (B). Control experiments (not shown) confirmed that CHX treatment completely inhibited cyclin B synthesis.

that cyclin E/Cdk2 is either part of or directly influenced by this timer.

The results presented here support the concept that the developmental timer is terminated at the MBT due to maternal cyclin degradation rather than initiation of zygotic transcription. An equally important question concerns how the timer is activated at fertilization. Unfertilized eggs are maintained in metaphase arrest through the action of cytostatic factor (CSF). CSF activity relies on the activity of the mos proto-oncogene kinase, an upstream regulator of the MAP kinase pathway (Sagata *et al.,* 1989; Roy *et al.,* 1996). Mos mRNA and protein are normally degraded during the first cell cycle after fertilization, but reexpression in cleavage divisions of mos, MAPK, or other elements of the pathway lead to metaphase arrest (Haccard *et al.,* 1993; Yew *et al.,* 1992; Huang *et al.,* 1995). Howe and Newport (1996) showed that mos or MAP kinase kinase expression in pre-MBT embryos blocked the degradation of cyclin E that would have occurred at the MBT. This effect on the developmental timer is unlikely to be a consequence of metaphase arrest per se, because the timer can operate without a mitotic cell cycle. MAPK-dependent arrest could simply stop the timer, preventing further developmental progression. An alternative possibility is that MAPK expression is sufficient to reset the timer to 0. Support for this idea has come from recent work by Vande Woude and associates (Murakami and Vande Woude, 1997). They confirmed earlier studies that showed the first cleavage cycle is 90 min in length and is characterized by wee1-dependent tyrosine phosphorylation of Cdc2. Interestingly, in blastomeres arrested by mos or MAPK, rerelease of metaphase arrest by calcium treatment restarts the cell cycle, but it now begins with features of the first cell cycle rather than continuation of the rapid, 22-min cycles of cleavage divisions 2–12. This suggests that MAPK can, indeed, reset the timer to 0 rather than simply stopping it.

An autonomous cytoplasmic oscillator that regulates the cell cycle independently of the nucleus was originally proposed based on the surface contraction waves of the fertilized or activated *Xenopus* egg (Hara *et al.,* 1980). Later, it was found that MPF (cyclin B/Cdc2) activity mirrored the surface contraction waves, and it was suggested that changes in this oscillator lead to the appearance of G phases after the MBT (Newport and Kirschner, 1982). At present it is clear that certain aspects of oscillator function occur even in the absence of MPF or a mitotic cell cycle, i.e., in the presence of CHX. Currently no components of the cytoplasmic oscillator or of the developmental timer that controls its expression have been identified. However, cyclin E/Cdk2 is an attractive candidate component of the cytoplasmic oscillator because it shares with the oscillator autonomous function in the absence of protein synthesis and the mitotic cell cycle and because it can affect the timing of early mitotic cell cycles and the MBT. The proposed developmental timer limits the expression of both the oscillator and cyclin E/Cdk2 activity, since after the MBT cyclin E is degraded and a simple oscillatory cell cycle is no longer evident.

It is not yet clear what event regulated by cyclin E/Cdk2 is monitored by the developmental timer. However, like cyclin E/Cdk2 activity, the centrosome duplication cycle is also independent of protein synthesis and the cell division cycle (Gard *et al.,* 1990; Sluder *et al.,* 1990). Centrosomes duplicate during S-phase and migrate to the spindle poles during mitosis, making components of the centrosome duplication pathway potential candidates for regulation by cyclin E/Cdk2 during the early cycles. It is possible that the centrosome duplication cycle can control the length of the early embryonic cell cycle. Whether cyclin E/Cdk2 has a role in centrosome duplication or whether the centrosome cycle is linked to the developmental timer remain important questions for further investigation.

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